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Sorge

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(54) **METHODS FOR DETECTION OF A TARGET NUCLEIC ACID SEQUENCE**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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(22) Filed: **Aug. 30, 2000**

Related U.S. Application Data

(63) Continuation-in-part of application No. 09/430,692, filed on Oct. 29, 1999.

(51) **Int. Cl.**[7] **C12Q 1/68**

(52) **U.S. Cl.** **435/6**; 435/6; 435/4; 435/18; 435/91.53; 435/183; 435/194; 435/195; 435/196; 435/810; 435/822; 436/94; 530/350

(58) **Field of Search** 435/6, 4, 18, 91.53, 435/183, 194, 195, 196, 810, 822; 436/94; 530/350

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,312,728	A	5/1994	Lizardi et al.	435/6
5,466,591	A *	11/1995	Abramson et al.	435/194
5,556,772	A	9/1996	Sorge et al.	435/91.2
5,843,669	A *	12/1998	Kaiser et al.	435/6
5,853,990	A *	12/1998	Winger et al.	435/6
5,874,283	A *	2/1999	Harrington et al.	435/252.3
5,985,557	A	11/1999	Prudent et al.	435/6
5,994,069	A	11/1999	Hull et al.	435/6

FOREIGN PATENT DOCUMENTS

WO	WO 98/23774	6/1998	

OTHER PUBLICATIONS

Lyamichev et al. Nature Biotechnology. vol. 17. Mar. 1999. pp. 292–296.*

Copy of International Search Report (PCT/US01/31670).

Lyamichev, et al, Structure–Specific Endonucleolytic Cleavage of Nucleic Acids by Eubacterial DNA Polymerases, *Science*, vol. 260, 1993, p. 778–783.

Lyamichev, et al, Polymorphism Identification And Quantitative Detection Of Genomic DNA by Invasive Cleavage Of Oligonucleotie Probes, Nature Biotechnology, vol. 17, 1999, p. 292–296.

Livak, et al, Oligonucleotides with Fluorescent Dyes at Opposite Ends Provide a Quenched Probe System Useful for Detecting PCR Product and Nucleic Acid Hybridization, *PCR Methods and Applications, Cold Harbor Laboratory Press*, 1995, p 357–362.

Copy of International search report. (PCT/US00/29663).

* cited by examiner

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(57) **ABSTRACT**

The invention relates to a method of generating a signal indicative of the presence of a target nucleic acid sequence in a sample, where the method includes forming a cleavage structure by incubating a sample containing a target nucleic acid sequence with a nucleic acid polymerase and cleaving the cleavage structure with a FEN nuclease to generate a cleaved nucleic acid fragment. The invention also relates to methods of detecting or measuring a target nucleic acid sequence, where the method includes forming a cleavage structure by incubating a target nucleic acid sequence with a nucleic acid polymerase, cleaving the cleavage structure with a FEN nuclease and detecting or measuring the release of a fragment.

29 Claims, 13 Drawing Sheets

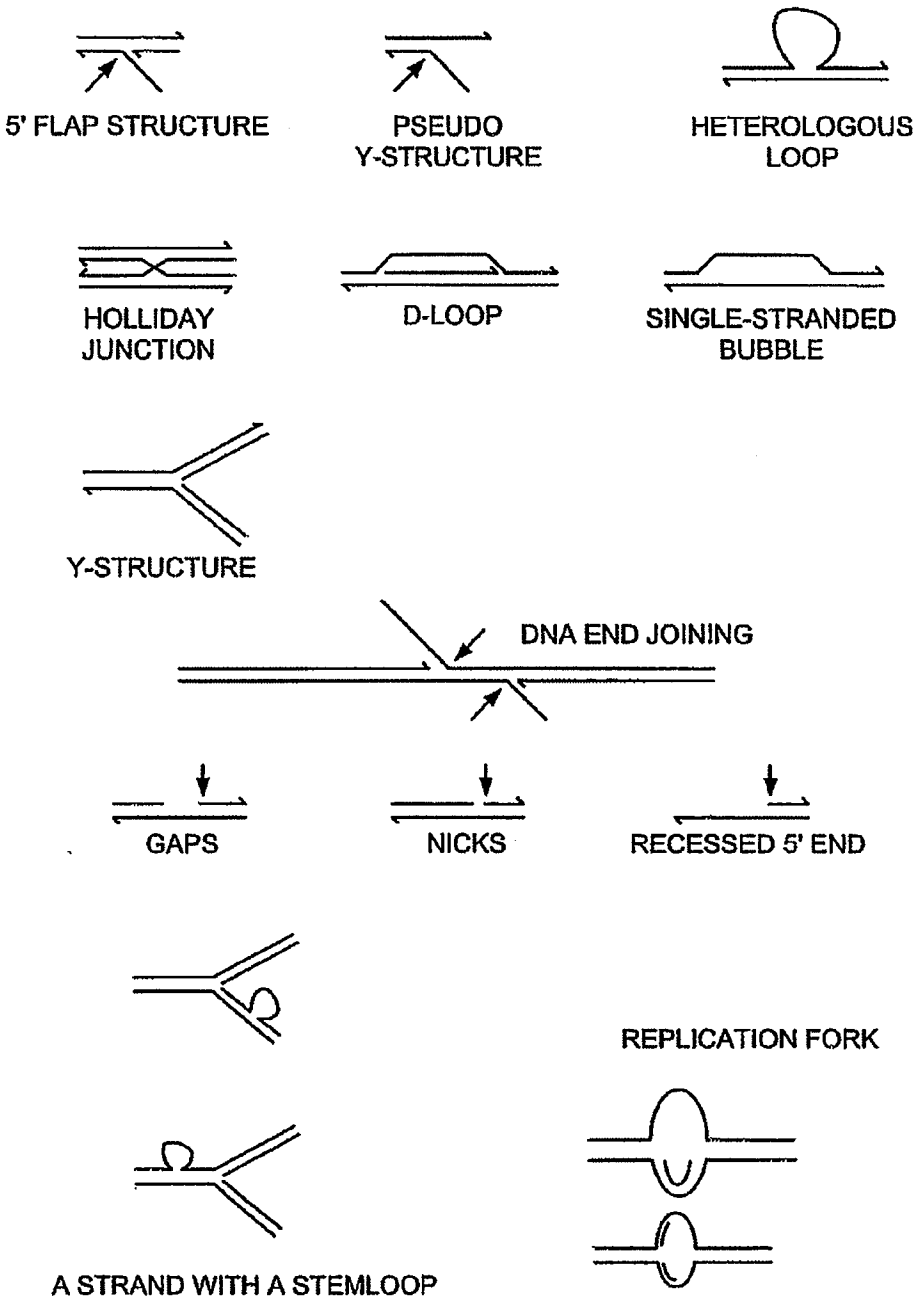
5' FLAP STRUCTURE
PSEUDO Y-STRUCTURE
HETEROLOGOUS LOOP
HOLLIDAY JUNCTION
D-LOOP
SINGLE-STRANDED BUBBLE
Y-STRUCTURE
DNA END JOINING
GAPS
NICKS
RECESSED 5' END
REPLICATION FORK
A STRAND WITH A STEMLOOP

FIG. 1

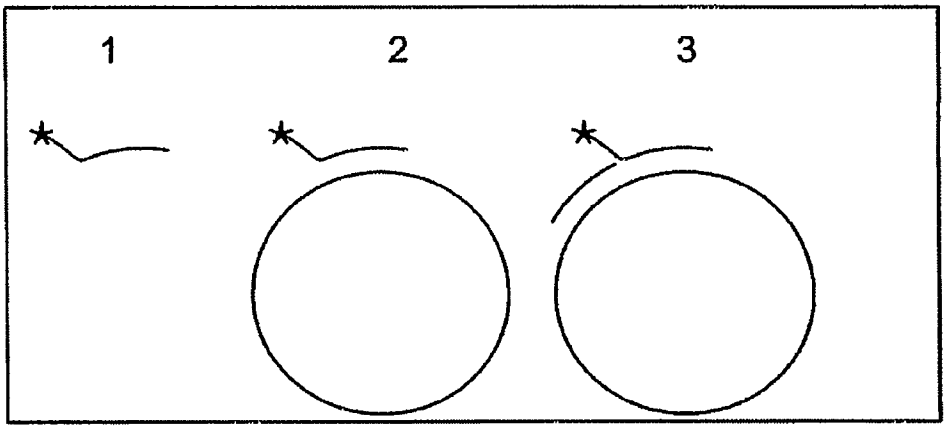

FIG. 2

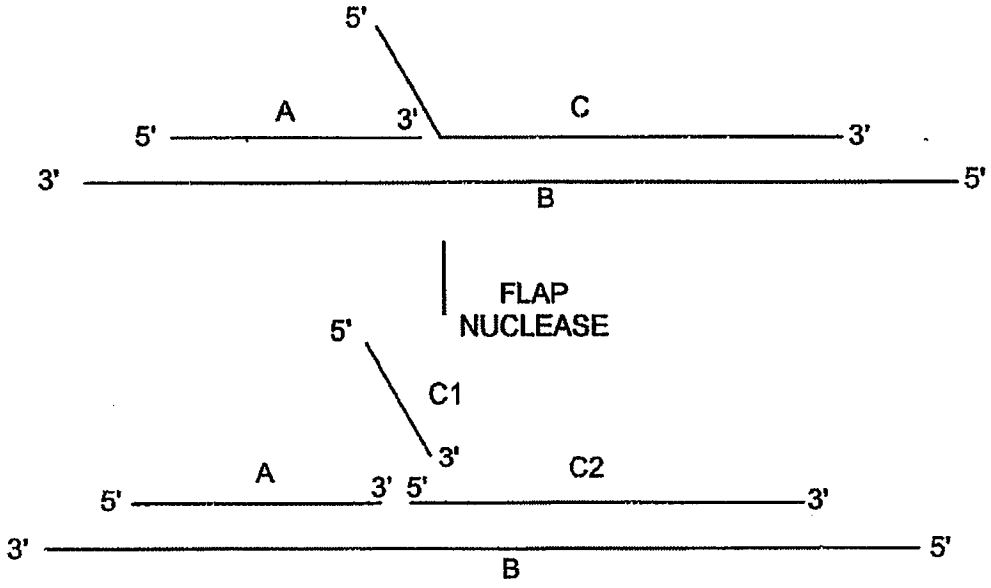

FIG. 3

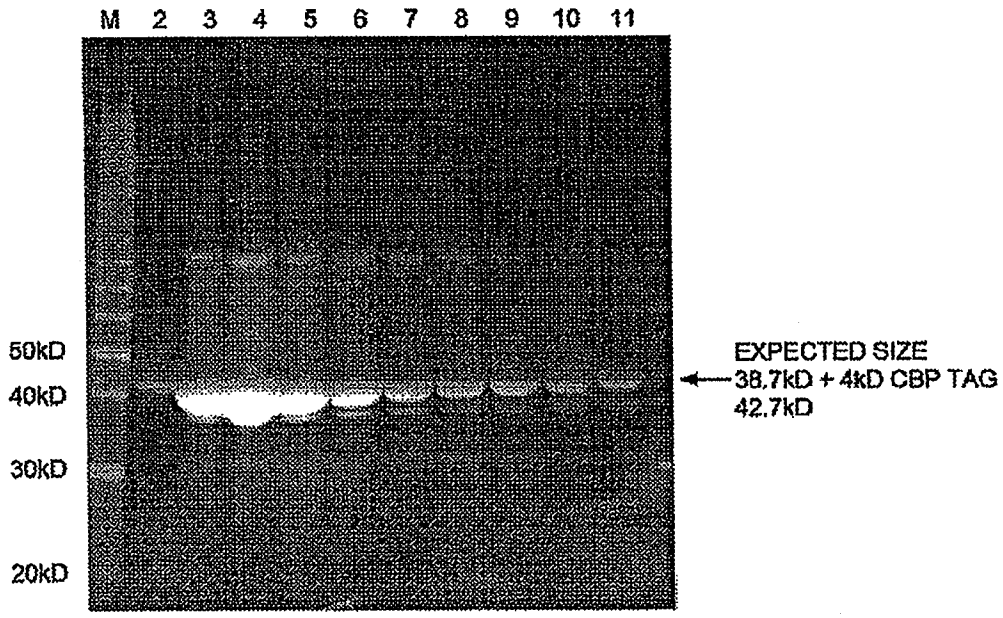
M 2 3 4 5 6 7 8 9 10 11
50kD
40kD
30kD
20kD
EXPECTED SIZE
38.7kD + 4kD CBP TAG
42.7kD

FIG. 4

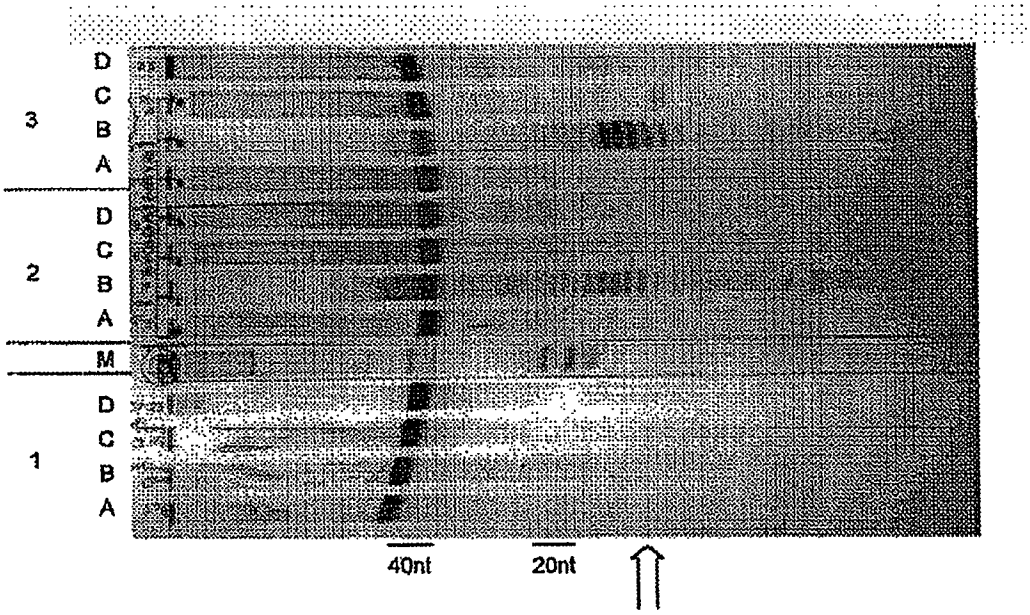

FIG. 5

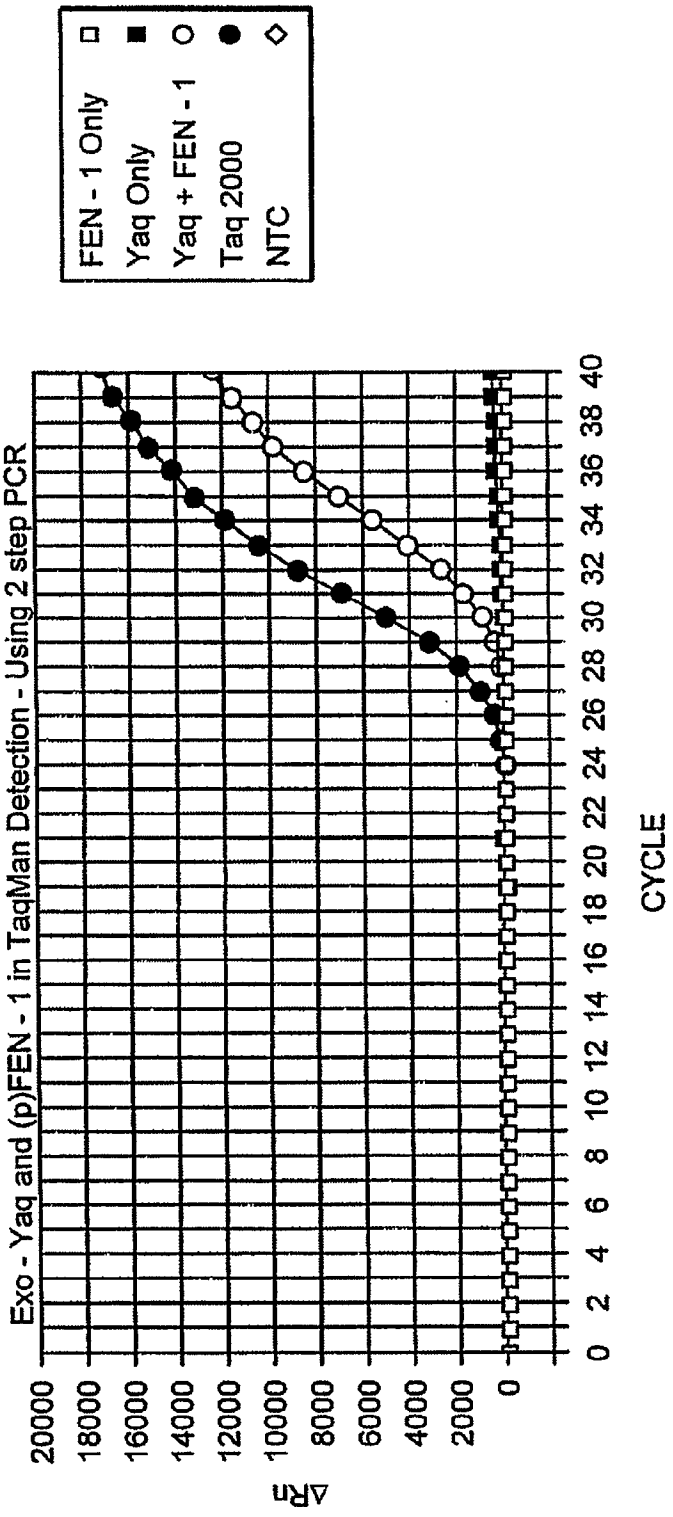
FEN - 1 Only
Yaq Only
Yaq + FEN - 1
Taq 2000
NTC
Exo - Yaq and (p)FEN - 1 in TaqMan Detection - Using 2 step PCR
ΔRn
CYCLE
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40

FIG. 6

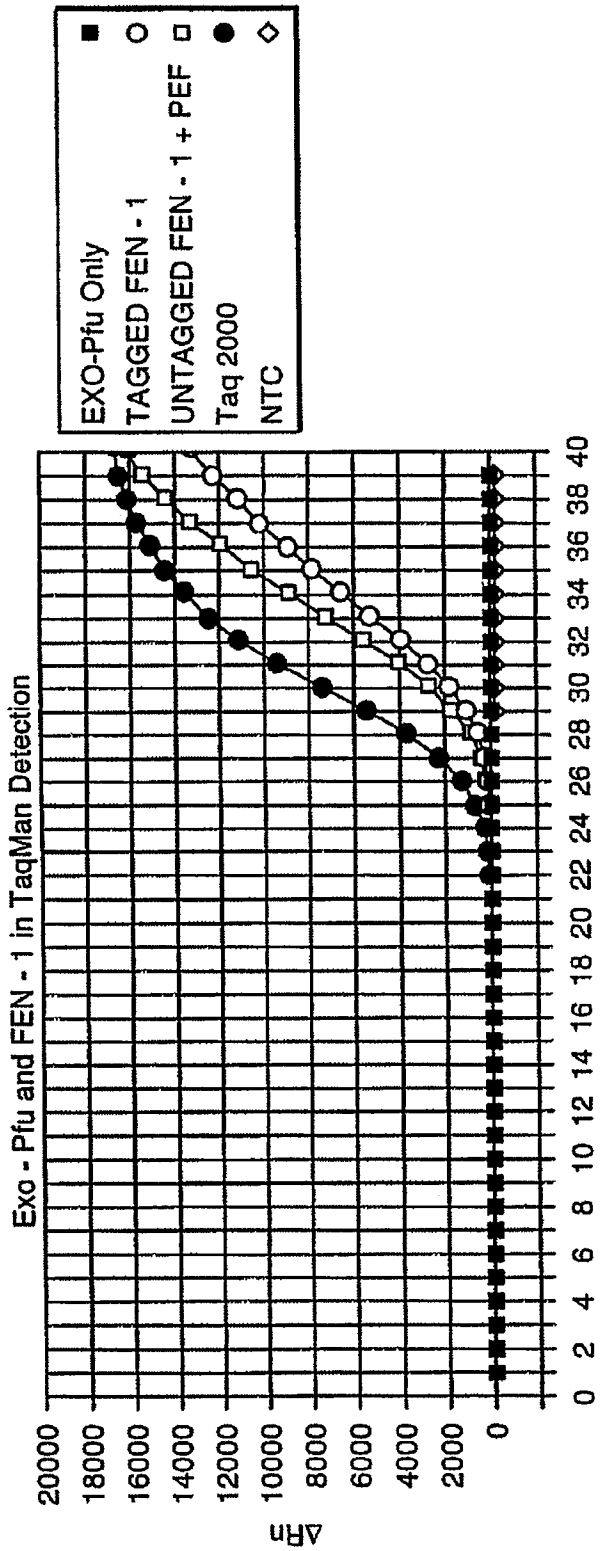
EXO-Pfu Only
TAGGED FEN - 1
UNTAGGED FEN - 1 + PEF
Taq 2000
NTC
Exo - Pfu and FEN - 1 in TaqMan Detection
ΔRn
CYCLE
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40

FIG. 7

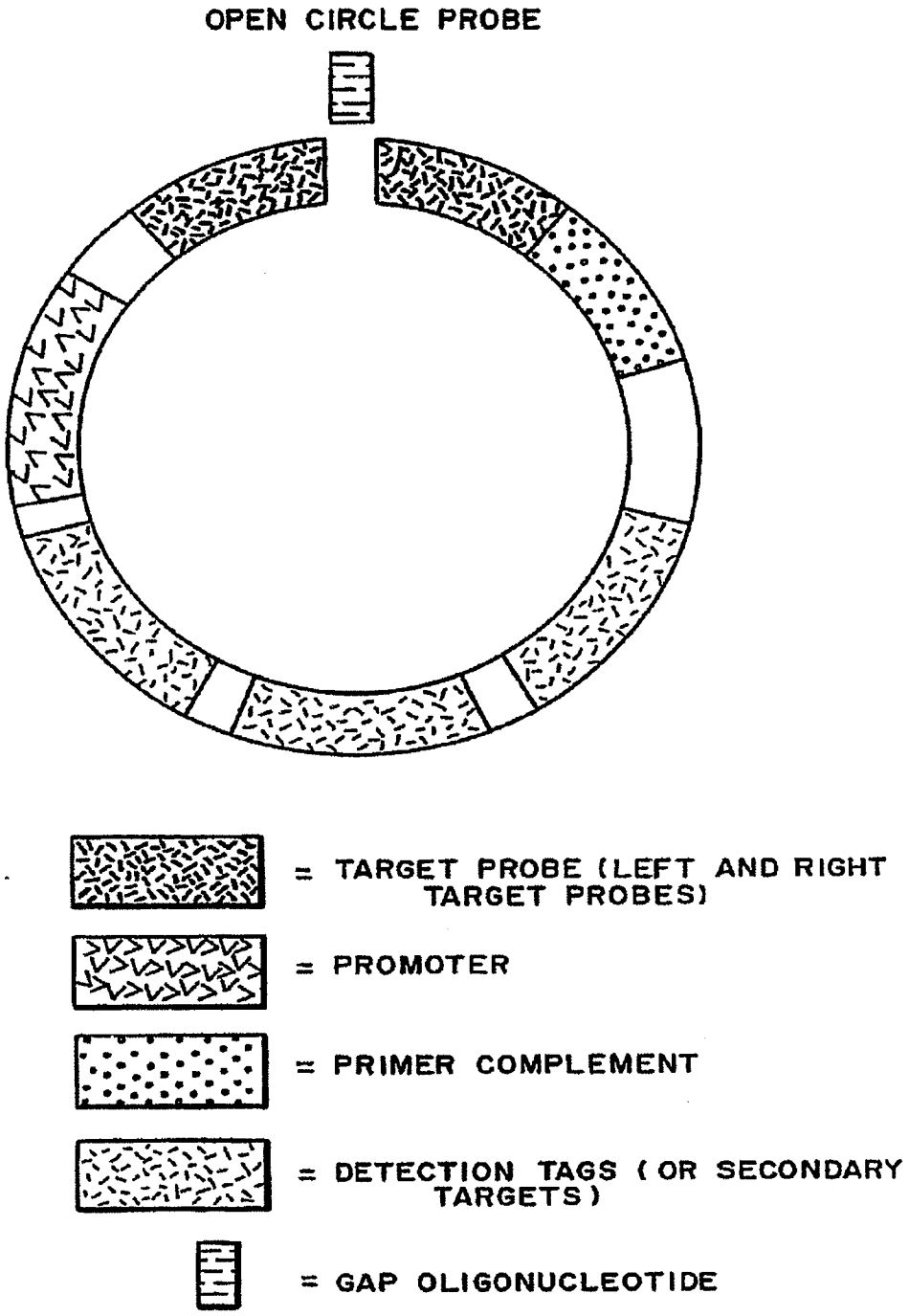
OPEN CIRCLE PROBE
= TARGET PROBE (LEFT AND RIGHT TARGET PROBES)
= PROMOTER
= PRIMER COMPLEMENT
= DETECTION TAGS (OR SECONDARY TARGETS)
= GAP OLIGONUCLEOTIDE

FIG. 8A

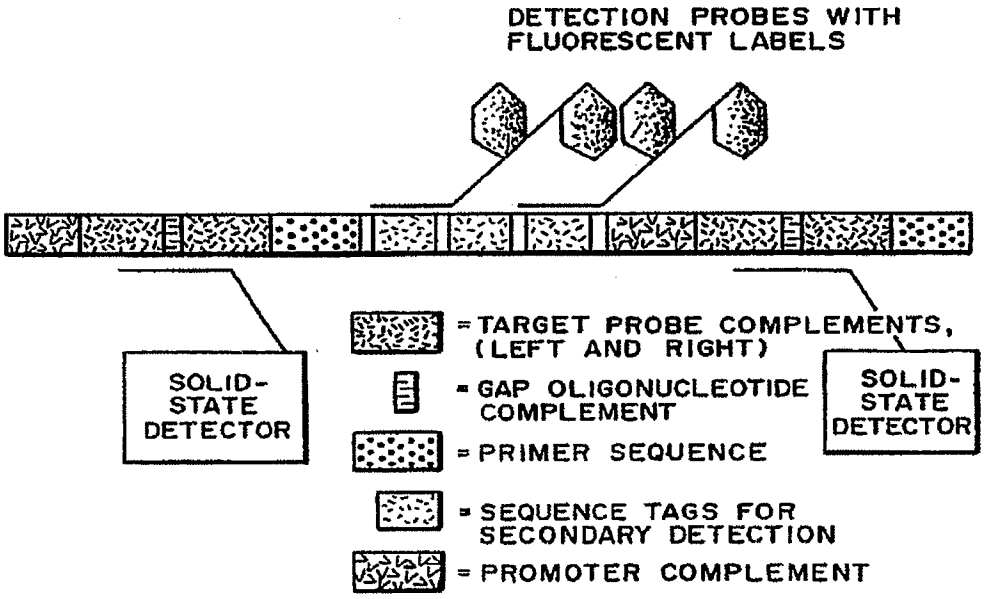
DETECTION PROBES WITH FLUORESCENT LABELS
SOLID-STATE DETECTOR
SOLID-STATE DETECTOR
= TARGET PROBE COMPLEMENTS, (LEFT AND RIGHT)
= GAP OLIGONUCLEOTIDE COMPLEMENT
= PRIMER SEQUENCE
= SEQUENCE TAGS FOR SECONDARY DETECTION
= PROMOTER COMPLEMENT

FIG. 8B

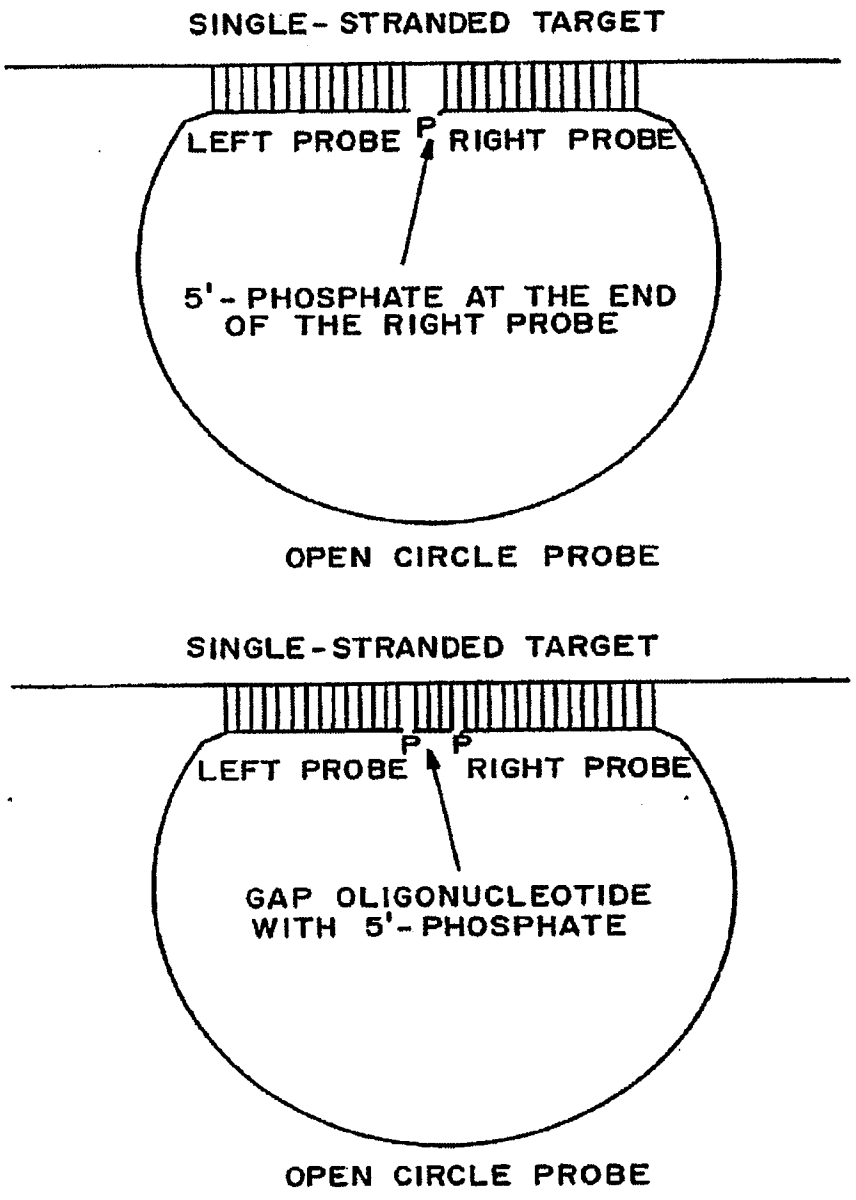
SINGLE-STRANDED TARGET
LEFT PROBE
P
RIGHT PROBE
5'-PHOSPHATE AT THE END OF THE RIGHT PROBE
OPEN CIRCLE PROBE
SINGLE-STRANDED TARGET
P
P
LEFT PROBE
RIGHT PROBE
GAP OLIGONUCLEOTIDE WITH 5'-PHOSPHATE
OPEN CIRCLE PROBE

FIG. 9A

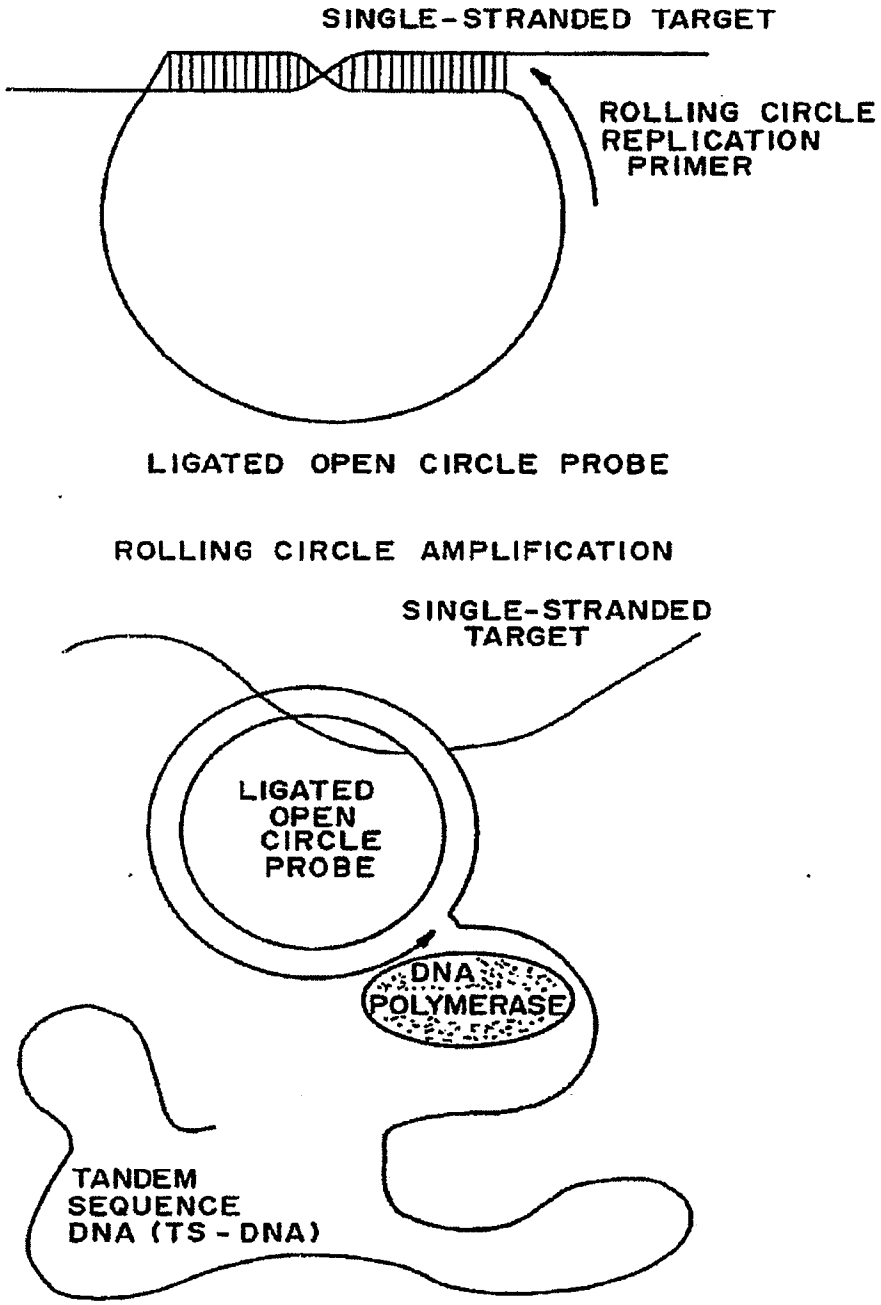
SINGLE-STRANDED TARGET
ROLLING CIRCLE REPLICATION PRIMER
LIGATED OPEN CIRCLE PROBE
ROLLING CIRCLE AMPLIFICATION
SINGLE-STRANDED TARGET
LIGATED OPEN CIRCLE PROBE
DNA POLYMERASE
TANDEM SEQUENCE DNA (TS - DNA)

FIG. 9B

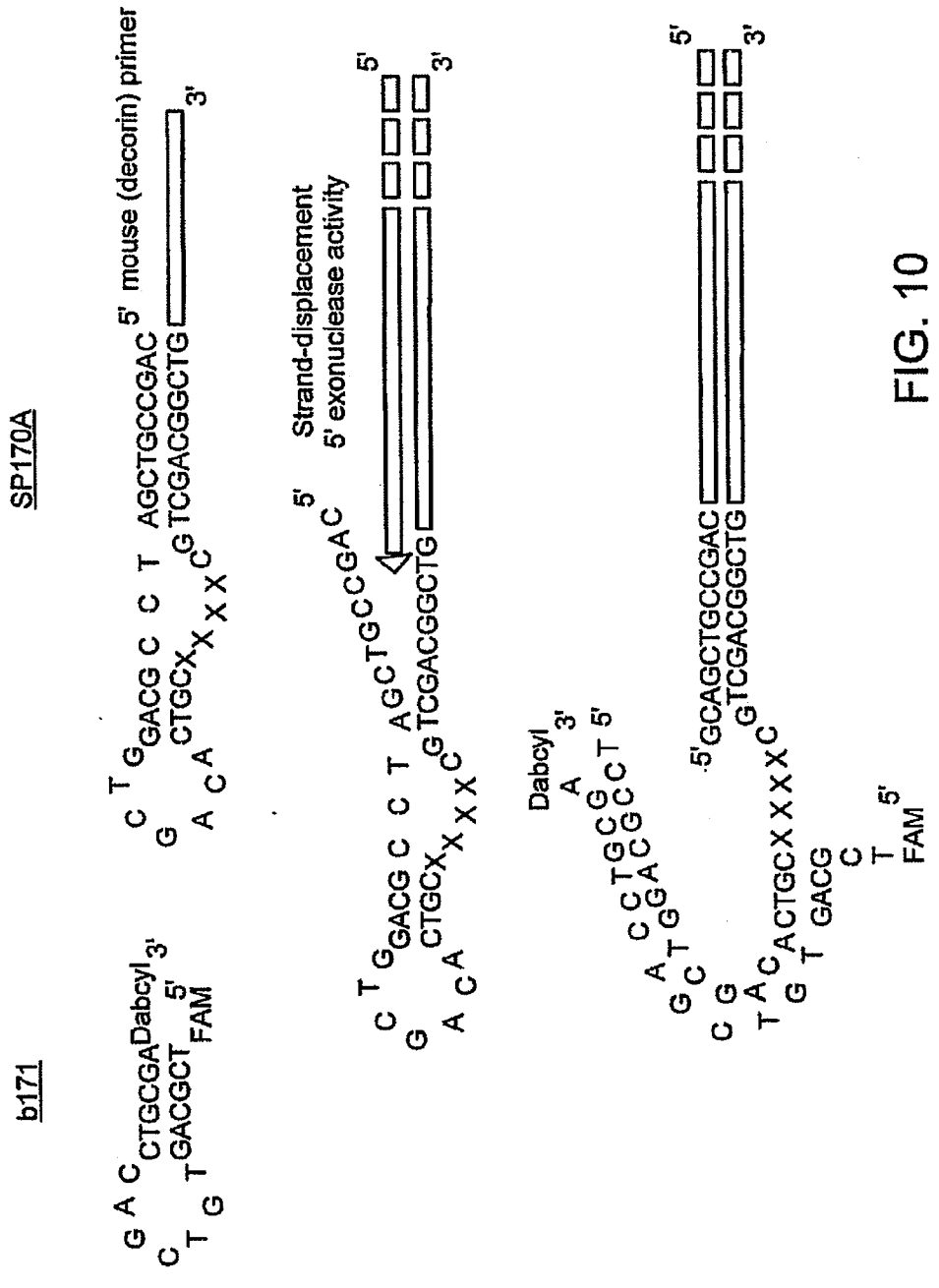
SP170A
b171
5' mouse (decorin) primer
Strand-displacement
5' exonuclease activity
Dabcyl
FAM

FIG. 10

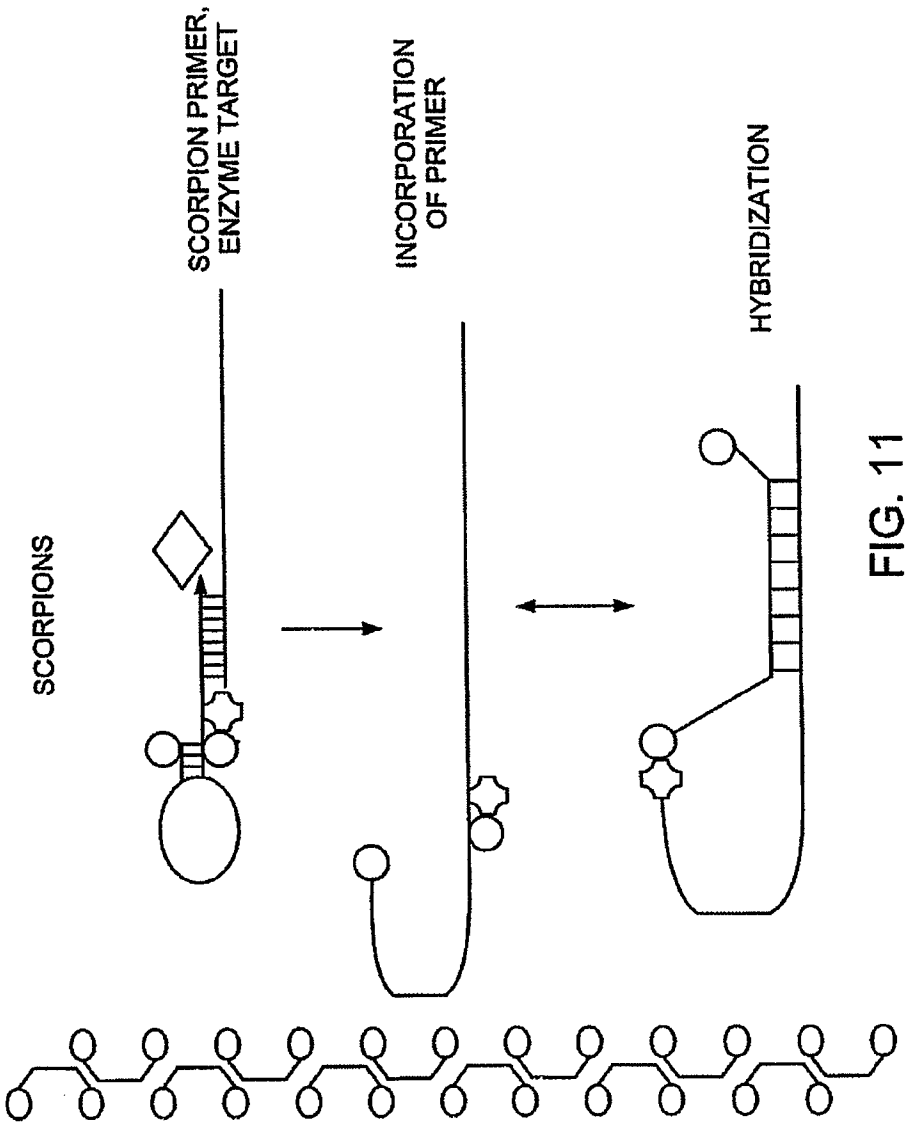
SCORPIONS
SCORPION PRIMER, ENZYME TARGET
INCORPORATION OF PRIMER
HYBRIDIZATION

FIG. 11

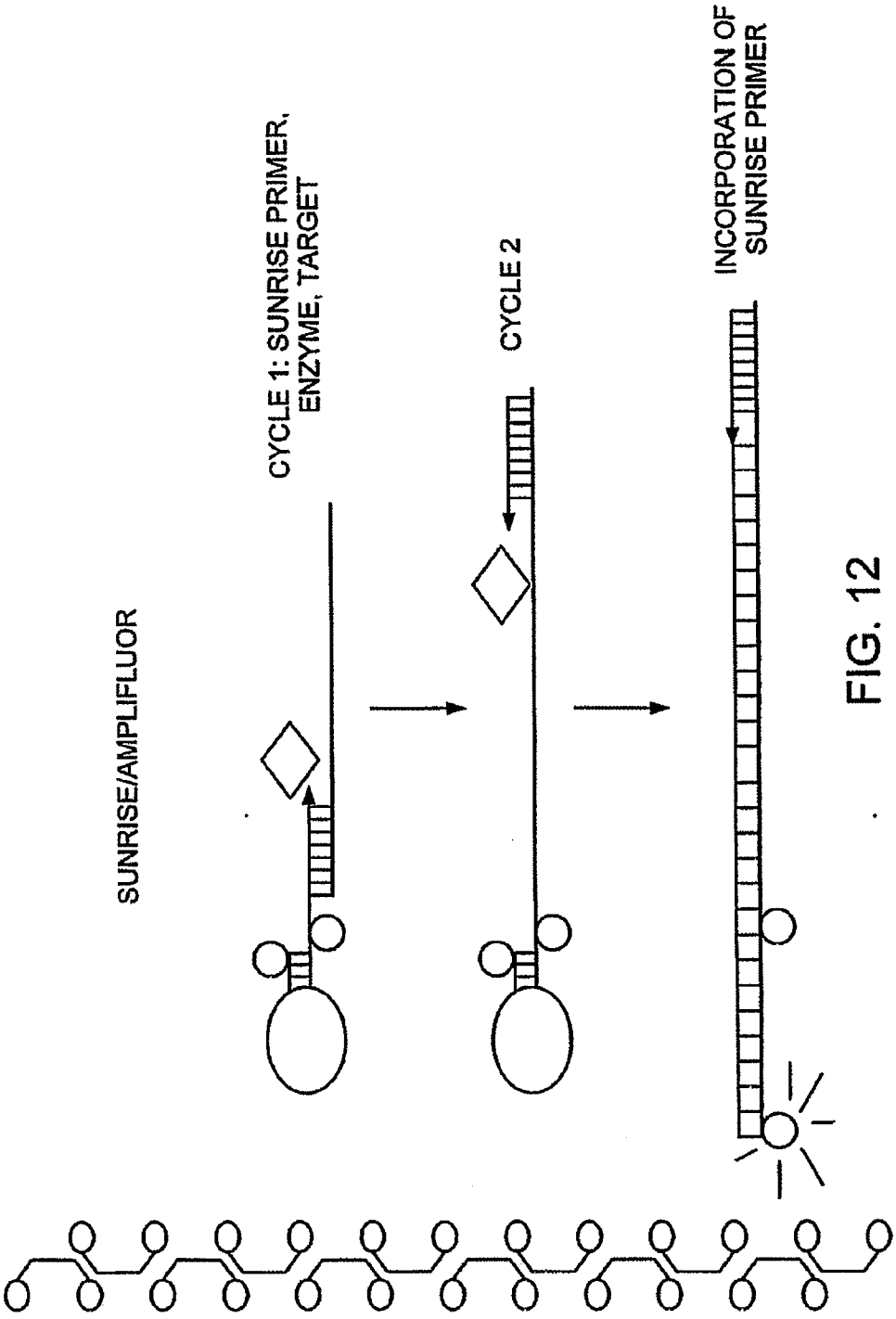
SUNRISE/AMPLIFLUOR
CYCLE 1: SUNRISE PRIMER, ENZYME, TARGET
CYCLE 2
INCORPORATION OF SUNRISE PRIMER

FIG. 12

METHODS FOR DETECTION OF A TARGET NUCLEIC ACID SEQUENCE

This application is a continuation-in-part of application Ser. No. 09/430,692, filed Oct. 29, 1999.

FIELD OF THE INVENTION

The invention relates in general to methods of detecting or measuring a target nucleic acid sequence.

BACKGROUND OF THE INVENTION

The fidelity of DNA replication, recombination, and repair is essential for maintaining genome stability, and all of these processes depend on 5'→3' exonuclease enzymes which are present in all organisms. For DNA repair, these enzymes are required for damaged fragment excision and recombinational mismatch correction. For replication, these nucleases are critical for the efficient processing of Okazaki fragments during lagging strand DNA synthesis. In *Escherichia coli*, this latter activity is provided by DNA polymerase I (PolI); *E. coli* strains with inactivating mutations in the PolI 5'→3' exonuclease domain are not viable due to an inability to process Okazaki fragments. Eukaryotic DNA polymerases, however, lack an intrinsic 5'→3' exonuclease domain, and this critical activity is provided by the multifunctional, structure-specific metallonuclease FEN-1 (five' exonuclease-1 or flap endonuclease-1), which also acts as an endonuclease for 5' DNA flaps (Reviewed in Hosfield et al., 1998a, *Cell*, 95:135).

Methods of detecting and/or measuring a nucleic acid wherein an enzyme produces a labeled nucleic acid fragment are known in the art.

U.S. Pat. Nos. 5,843,669, 5,719,028, 5,837,450, 5,846, 717 and 5,888,780 disclose a method of cleaving a target DNA molecule by incubating a 5' labeled target DNA with a DNA polymerase isolated from *Thermus aquaticus* (Taq polymerase) and a partially complementary oligonucleotide capable of hybridizing to sequences at the desired point of cleavage. The partially complementary oligonucleotide directs the Taq polymerase to the target DNA through formation of a substrate structure containing a duplex with a 3' extension opposite the desired site of cleavage wherein the non-complementary region of the oligonucleotide provides a 3' arm and the unannealed 5' region of the substrate molecule provides a 5' arm. The partially complementary oligonucleotide includes a 3' nucleotide extension capable of forming a short hairpin. The release of labeled fragment is detected following cleavage by Taq polymerase.

U.S. Pat. Nos. 5,843,669, 5,719,028, 5,837,450, 5,846, 717 and 5,888,780 disclose the generation of mutant, thermostable DNA polymerases that have very little or no detectable synthetic activity, and wild type thermostable nuclease activity. The mutant polymerases are said to be useful because they lack 5' to 3' synthetic activity; thus synthetic activity is an undesirable side reaction in combination with a DNA cleavage step in a detection assay.

U.S. Pat. Nos. 5,843,669, 5,719,028, 5,837,450, 5,846, 717 and 5,888,780 disclose that wild type Taq polymerase or mutant Taq polymerases that lack synthetic activity can release a labeled fragment by cleaving a 5' end labeled hairpin structure formed by heat denaturation followed by cooling, in the presence of a primer that binds to the 3' arm of the hairpin structure. Further, U.S. Pat. Nos 5,843,669, 5,719,028, 5,837,450, 5,846,717 and 5,888,780 teach that the mutant Taq polymerases lacking synthetic activity can also cleave this hairpin structure in the absence of a primer that binds to the 3' arm of the hairpin structure.

U.S. Pat. Nos. 5,843,669, 5,719,028, 5,837,450, 5,846, 717 and 5,888,780 also disclose that cleavage of this hairpin structure in the presence of a primer that binds to the 3' arm of the hairpin structure by mutant Taq polymerases lacking synthetic activity yields a single species of labeled cleaved product, while wild type Taq polymerase produces multiple cleavage products and converts the hairpin structure to a double stranded form in the presence of dNTPs, due to the high level of synthetic activity of the wild type Taq enzyme.

The 5' to 3' exonuclease activity of a nucleic acid polymerase can impair the amplification of certain nucleic acids. There is also a need in the art for a method of generating a signal using a nucleic acid cleavage reaction in the absence of a 5' to 3' exonuclease activity of a nucleic acid polymerase.

U.S. Pat Nos. 5,843,669, 5,719,028, 5,837,450, 5,846, 717 and 5,888,780 also disclose that mutant Taq polymerases exhibiting reduced synthetic activity, but not wild type Taq polymerase, can release a single labeled fragment by cleaving a linear nucleic acid substrate comprising a 5' end labeled target nucleic acid and a complementary oligonucleotide wherein the complementary oligonucleotide hybridizes to a portion of the target nucleic acid such that 5' and 3' regions of the target nucleic acid are not annealed to the oligonucleotide and remain single stranded.

There is a need in the art for a method of generating a signal of a discrete size that can be easily distinguished from oligonucleotide fragments that may arise from nuclease contaminants, using a nucleic acid cleavage reaction in the absence of 5' to 3' exonuclease activity of a nucleic acid polymerase.

U.S. Pat. Nos. 5,843,669, 5,719,028, 5,837,450, 5,846, 717 and 5,888,780 also disclose a method of cleaving a labeled nucleic acid substrate at naturally occurring areas of secondary structure. According to this method, biotin labeled DNA substrates are prepared by PCR, mixed with wild type Taq polymerase or CleavaseBN (a mutant Taq polymerase with reduced synthetic activity and wild type 5' to 3' nuclease activity), incubated at 95° C. for 5 seconds to denature the substrate and then quickly cooled to 65° C. to allow the DNA to assume its unique secondary structure by allowing the formation of intra-strand hydrogen bonds between the complementary bases. The reaction mixture is incubated at 65° C. to allow cleavage to occur and biotinylated cleavage products are detected.

There is a need in the art for a method of generating a signal using a nucleic acid cleavage reaction in the absence of a 5' to 3' exonuclease activity of a nucleic acid polymerase wherein the cleavage structure is not required to contain areas of secondary structure

Methods of detecting and/or measuring a nucleic acid wherein a FEN-1 enzyme is used to generate a labeled nucleic acid fragment are known in the art.

U.S. Pat. No. 5,843,669 discloses a method of detecting polymorphisms by cleavase fragment length polymorphism analysis using a thermostable FEN-1 nuclease in the presence or absence of a mutant Taq polymerase exhibiting reduced synthetic activity. According to this method, double stranded Hepatitis C virus (HCV) DNA fragments are labeled by using 5' end labeled primers (labeled with TMR fluorescent dye) in a PCR reaction The TMR labeled PCR products are denatured by heating to 95° C. and cooled to 55° C. to generate a cleavage structure. U.S. Pat. No. 5,843,669 discloses that a cleavage structure comprises a region of a single stranded nucleic acid substrate containing secondary structure. Cleavage is carried out in the presence

of CleavaseBN nuclease, FEN-1 nuclease derived from the archaebacteria *Methanococcus jannaschii* or both enzymes. Labeled reaction products are visualized by gel electrophoresis followed by fluoroimaging. U.S. Pat. No 5,843,669 discloses that CleavaseBN nuclease and *Methanococcus jannaschii* FEN-1 nuclease produce cleavage patterns that are easily distinguished from each other, and that the cleavage patterns from a reaction containing both enzymes include elements of the patterns produced by cleavage with each individual enzyme but are not merely a composite of the cleavage patterns produced by each individual enzyme. This indicates that some of the fragments that are not cleaved by one enzyme (and which appear as a band in that enzyme's pattern) can be cleaved by a second enzyme in the same reaction mixture.

Lyamichev et al. disclose a method for detecting DNAs wherein overlapping pairs of oligonucleotide probes that are partially complementary to a region of target DNA are mixed with the target DNA to form a 5' flap region, and wherein cleavage of the labeled downstream probe by a thermostable FEN-1 nuclease produces a labeled cleavage product. Lyamichev et al. also disclose reaction conditions wherein multiple copies of the downstream oligonucleotide probe can be cleaved for a single target sequence in the absence of temperature cycling, so as to amplify the cleavage signal and allow quantitative detection of target DNA at sub-attomole levels (Lyamichev et al., 1999, *Nat. Biotechnol*, 17:292).

The polymerase chain reaction (PCR) technique, is disclosed in U.S. Pat. Nos. 4,683,202, 4,683,195 and 4,800,159. In its simplest form, PCR is an in vitro method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of reaction steps involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. PCR is reported to be capable of producing a selective enrichment of a specific DNA sequence by a factor of 10^9. The PCR method is also described in Saiki et al., 1985, *Science*, 230:1350.

While the PCR technique is an extremely powerful method for amplifying nucleic acid sequences, the detection of the amplified material requires additional manipulation and subsequent handling of the PCR products to determine whether the target DNA is present. It is desirable to decrease the number of subsequent handling steps currently required for the detection of amplified material. An assay system, wherein a signal is generated while the target sequence is amplified, requires fewer handling steps for the detection of amplified material, as compared to a PCR method that does not generate a signal during the amplification step.

U.S. Pat. Nos. 5,210,015 and 5,487,972 disclose a PCR based assay for releasing labeled probe comprising generating a signal during the amplification step of a PCR reaction in the presence of a nucleic acid to be amplified, Taq polymerase that has 5' to 3' exonuclease activity and a 5', 3' or 5' and 3' end-labeled probe comprising a region complementary to the amplified region and an additional non-complementary 5' tail region. U.S. Pat. Nos. 5,210,015 and 5,487,972 disclose further that this PCR based assay can liberate the 5' labeled end of a hybridized probe when the Taq polymerase is positioned near the labeled probe by an upstream probe in a polymerization independent manner, e.g. in the absence of dNTPs.

There is a need in the art for a method of detecting or measuring a target nucleic acid sequence that does not require multiple steps.

There is also a need in the art for a PCR process for detecting or measuring a target nucleic acid sequence that does not require multiple steps subsequent to the amplification process

There is also a need in the art for a PCR process for detecting or measuring a target nucleic acid sequence that allows for concurrent amplification and detection of a target nucleic acid sequence in a sample.

There is also a need in the art for a PCR process for detecting or measuring a target nucleic acid sequence wherein the PCR process occurs in the presence of a nucleic acid polymerase that lacks 5' to 3' exonuclease activity.

SUMMARY OF THE INVENTION

The invention provides a method of generating a signal indicative of the presence of a target nucleic acid sequence in a sample comprising forming a cleavage structure by incubating a sample comprising a target nucleic acid sequence with a nucleic acid polymerase, and cleaving the cleavage structure with a FEN nuclease to generate a signal, wherein generation of the signal is indicative of the presence of a target nucleic acid sequence in the sample.

As used herein a "FEN nuclease" refers to an enzyme that cleaves a cleavage structure according to the invention. The term "FEN nuclease" encompasses an enzyme that consists essentially of a 5' exonuclease and/or an endonuclease activity. As used herein, "consists essentially of" refers to an enzyme wherein the predominant activity of the enzyme is a 5' exonucleolytic and/or endonucleolytic activity, such that one or both of 5' to 3' synthetic activity and 3' single-stranded flap cleavage activity (i.e., 3' endonucleolytic and/or 3'exonucleolytic activity) are substantially lacking. "Substantially lacks" means that the FEN nuclease possesses no more than 5% or 10% and preferably less than 0.1%, 0.5%, or 1% of the activity of a wild type enzyme (e.g. for 5' to 3' synthetic activity and the 3' endonucleolytic and/or '3 exonucleolytic activities, the enzyme may be a wild type DNA polymerase having these activities). 5' to 3' synthetic activity can be measured, for example, in a nick translation assay or an enzymatic sequencing reaction which involve the formation of a phosphodiester bridge between the 3'-hydroxyl group at the growing end of an oligonucleotide primer and the 5'-phosphate group of an incoming deoxynucleotide, such that the overall direction of synthesis is in the 5' to 3' direction 3' flap cleavage may be measured in a DNA synthesis reaction in which, because the (labeled) 3' end of a DNA duplex is unpaired, it is cleaved from the duplex. A FEN nuclease that "consists of" a 5' exonuclease and/or endonuclease activity refers to an enzyme that "lacks" 5' to 3' synthetic activity and/or 3' single-stranded flap cleavage activity. "Lacks" means that the Fen nuclease has no detectable activity or has only "minor" activity, i.e., less than 1.0%, 0.5%, 0.1% or 0.01% of the activity of a wild type enzyme. As used herein, "FEN nuclease" encompasses a 5' flap-specific nuclease.

As used herein, "wild type" refers to a gene or gene product which has the characteristics of (i.e., either has the sequence of or encodes, for the gene, or possesses the sequence or activity of, for an enzyme) that gene or gene product when isolated from a naturally occurring source.

A "5' flap-specific nuclease" (also referred to herein as a "flap-specific nuclease") according to the invention is an endonuclease which can remove a single stranded flap that protrudes as a 5' single strand. A flap-specific nuclease according to the invention can also cleave a pseudo-Y structure. A substrate of a flap-specific nuclease according to

the invention, comprises a target nucleic acid, a second nucleic acid, a portion of which specifically hybridizes with a target nucleic acid, and a primer extension product from a third nucleic acid that specifically hybridizes with a target nucleic acid sequence.

As used herein, a "cleavage structure" refers to a polynucleotide structure (for example as illustrated in FIG. 1) comprising at least a duplex nucleic acid having a single stranded region comprising a flap, a loop, a single-stranded bubble, a D-loop, a nick or a gap. A cleavage structure according to the invention thus includes a polynucleotide structure comprising a flap strand of a branched DNA wherein a 5' single-stranded polynucleotide flap extends from a position near its junction to the double stranded portion of the structure and preferably the flap is labeled with a detectable label. A flap of a cleavage structure according to the invention is preferably about 1–500 nucleotides, more preferably about 5–25 nucleotides and most preferably about 10–20 nucleotides and is preferably cleaved at a position located either one nucleotide proximal and/or one nucleotide distal from the elbow of the flap strand.

A cleavage structure according to the invention preferably comprises a target nucleic acid sequence, and also may include an oligonucleotide that specifically hybridizes with the target nucleic acid sequence, and a flap extending from the hybridizing oligonucleotide. For example, a cleavage structure according to the invention may comprise a target nucleic acid sequence (for example B in FIG. 3), an upstream oligonucleotide that is complementary to the target sequence (for example A in FIG. 3), and a downstream oligonucleotide that is complementary to the target sequence (for example C in FIG. 3). In such a cleavage structure, the downstream oligonucleotide may be blocked at the 3' terminus to prevent extension of the 3' end of the downstream oligonucleotide.

A cleavage structure according to the invention may be a polynucleotide structure comprising a flap extending from the downstream oligonucleotide, wherein the flap is formed by extension of the upstream oligonucleotide by the synthetic activity of a nucleic acid polymerase, and subsequent, partial, displacement of the 5' end of the downstream oligonucleotide.

A cleavage structure according to the invention may be formed by hybridizing a target nucleic acid sequence with an oligonucleotide wherein the oligonucleotide comprises a complementary region that anneals to the target nucleic acid sequence, and a non-complementary region that does not anneal to the target nucleic acid sequence and forms a 5' flap.

A cleavage structure also may be a pseudo-Y structure wherein a pseudo Y-structure is formed if the strand upstream of a flap (referred to herein as a flap adjacent strand or primer strand) is removed, and double stranded DNA substrates containing a gap or nick. A "cleavage structure", as used herein, does not include a double stranded nucleic acid structure with only a 3' single-stranded flap. As used herein, a "cleavage structure" comprises ribonucleotides or deoxyribonucleotides and thus can be RNA or DNA.

A cleavage structure according to the invention may be an overlapping flap wherein the 3' end of an upstream oligonucleotide capable of hybridizing to a target nucleic acid sequence (for example A in FIG. 3) is complementary to 1 base pair of the downstream oligonucleotide (for example C in FIG. 3) that is annealed to a target nucleic acid sequence and wherein the overlap is directly downstream of the point of extension of the single stranded flap.

A cleavage structure according to the invention is formed by the steps of 1. incubating a) an upstream extendable 3' end, preferably an oligonucleotide primer, b) an oligonucleotide primer probe located not more than 5000 nucleotides downstream of the upstream primer and c) an appropriate target nucleic acid sequence wherein the target sequence is complementary to both the upstream primer and downstream probe and d) a suitable buffer, under conditions that allow the nucleic acid sequence to hybridize to the oligonucleotide primers, and 2. extending the 3' end of the upstream oligonucleotide primer by the synthetic activity of a polymerase such that the newly synthesized 3' end of the upstream oligonucleotide primer becomes adjacent to and/or displaces at least a portion of (i e., at least 5–10 nucleotides of) the 5' end of the downstream oligonucleotide probe. According to the method of the invention, buffers and extension temperatures are favorable for strand displacement by a particular nucleic acid polymerase according to the invention. Preferably, the downstream oligonucleotide is blocked at the 3' terminus to prevent extension of the 3' end of the downstream oligonucleotide. In another embodiment of the invention, a cleavage structure according to the invention can be prepared by incubating a target nucleic acid sequence with an oligonucleotide primer comprising a non-complementary 5' region that does not anneal to the target nucleic acid sequence and forms a 5' flap, and a complementary 3' region that anneals to the target nucleic acid sequence.

In a preferred embodiment of the invention a cleavage structure is labeled. A labeled cleavage structure according to the invention is formed by the steps of 1. incubating a) an upstream extendable 3' end, preferably an oligonucleotide primer, b) a labeled probe preferably located not more than 5000 and more preferably located not more than 500 nucleotides downstream of the upstream primer and c) an appropriate target nucleic acid sequence wherein the target sequence is complementary to both the primer and the labeled probe and d) a suitable buffer, under conditions that allow the nucleic acid sequence to hybridize to the primers, and 2. extending the 3' end of the upstream primer by the synthetic activity of a polymerase such that the newly synthesized 3' end of the upstream primer partially displaces the 5' end of the downstream probe. According to the method of the invention, buffers and extension temperatures are favorable for strand displacement by a particular nucleic acid polymerase according to the invention Preferably, the downstream oligonucleotide is blocked at the 3' terminus to prevent extension of the 3' end of the downstream oligonucleotide. In another embodiment, a cleavage structure according to the invention can be prepared by incubating a target nucleic acid sequence with a probe comprising a non-complementary, labeled, 5' region that does not anneal to the target nucleic acid sequence and forms a 5' flap, and a complementary 3' region that anneals to the target nucleic acid sequence.

As used herein, "label" or "labeled moiety capable of providing a signal" refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can be operatively linked to a nucleic acid. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, mass spectrometry, binding affinity, hybridization radiofrequency and the like.

As used herein, "generating a signal" refers to detecting and or measuring a released nucleic acid fragment as an indication of the presence of a target nucleic acid sequence in a sample.

As used herein, "sample" refers to any substance containing or presumed to contain a nucleic acid of interest (a target nucleic acid sequence) or which is itself a nucleic acid containing or presumed to contain a target nucleic acid sequence of interest. The term "sample" thus includes a sample of nucleic acid (genomic DNA, cDNA, RNA), cell, organism, tissue, fluid, or substance including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, synovial fluid, urine, tears, stool, external secretions of the skin, respiratory, intestinal and genitourinary tracts, saliva, blood cells, tumors, organs, tissue, samples of in vitro cell culture constituents, natural isolates (such as drinking water, seawater, solid materials), microbial specimens, and objects or specimens that have been "marked" with nucleic acid tracer molecules

As used herein, "target nucleic acid sequence" or "template nucleic acid sequence" refers to a region of a nucleic acid that is to be either replicated, amplified, and/or detected. In one embodiment, the "target nucleic acid sequence" or "template nucleic acid sequence" resides between two primer sequences used for amplification.

As used herein, "nucleic acid polymerase" refers to an enzyme that catalyzes the polymerization of nucleoside triphosphates. Generally, the enzyme will initiate synthesis at the 3'-end of the primer annealed to the target sequence, and will proceed in the 5'-direction along the template, and if possessing a 5' to 3' nuclease activity, hydrolyzing intervening, annealed probe to release both labeled and unlabeled probe fragments, until synthesis terminates. Known DNA polymerases include, for example, *E. coli* DNA polymerase I, T7 DNA polymerase, *Thermus thermophilus* (Tth) DNA polymerase, *Bacillus stearothermophilus* DNA polymerase, *Thermococcus litoralis* DNA polymerase, *Thermus aquaticus* (Taq) DNA polymerase and *Pyrococcus furiosus* (Pfu) DNA polymerase.

As used herein, "5' to 3' exonuclease activity" or "5'→3' exonuclease activity" refers to that activity of a template-specific nucleic acid polymerase e.g. a 5'→3' exonuclease activity traditionally associated with some DNA polymerases whereby mononucleotides or oligonucleotides are removed from the 5' end of a polynucleotide in a sequential manner, (i.e., *E. coli* DNA polymerase I has this activity whereas the Klenow (Klenow et al., 1970, *Proc. Natl. Acad. Sci., USA*, 65:168) fragment does not, (Klenow et al., 1971, *Eur. J. Biochem.*, 22:371)), or polynucleotides are removed from the 5' end by an endonucleolytic activity that may be inherently present in a 5' to 3' exonuclease activity.

As used herein, the phrase "substantially lacks 5' to 3' exonuclease activity" or "substantially lacks 5'→3' exonuclease activity" means having less than 10%, 5%, 1%, 0.5%, or 0.1% of the activity of a wild type enzyme. The phrase "lacking 5' to 3' exonuclease activity" or "lacking 5'→3' exonuclease activity" means having undetectable 5' to 3' exonuclease activity or having less than about 1%, 0.5%, or 0.1% of the 5' to 3' exonuclease activity of a wild type enzyme. 5' to 3' exonuclease activity may be measured by an exonuclease assay which includes the steps of cleaving a nicked substrate in the presence of an appropriate buffer, for example 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 50 μg/ml bovine serum albumin) for 30 minutes at 60° C., terminating the cleavage reaction by the addition of 95% formamide containing 10 mM EDTA and 1 mg/ml bromophenol blue, and detecting nicked or un-nicked product.

Nucleic acid polymerases useful according to the invention include but are not limited to Pfu, exo– Pfu (a mutant form of Pfu that lacks 3' to 5' exonuclease activity), the Stoffel fragment of Taq, N-truncated Bst, N-truncated Bca, Genta, JdF3 exo–, Vent, Vent exo– (a mutant form of Vent that lacks 3' to 5' exonuclease activity), Deep Vent, Deep Vent exo– (a mutant form of Deep Vent that lacks 3' to 5' exonuclease activity), UlTma and Sequenase. Additional nucleic acid polymerases useful according to the invention are included below in the section entitled, "Nucleic Acid Polymerases".

As used herein, "cleaving" refers to enzymatically separating a cleavage structure into distinct (i.e. not physically linked to other fragments or nucleic acids by phosphodiester bonds) fragments or nucleotides and fragments that are released from the cleavage structure. For example, cleaving a labeled cleavage structure refers to separating a labeled cleavage structure according to the invention and defined below, into distinct fragments including fragments derived from an oligonucleotide that specifically hybridizes with a target nucleic acid sequence or wherein one of the distinct fragments is a labeled nucleic acid fragment derived from a target nucleic acid sequence and/or derived from an oligonucleotide that specifically hybridizes with a target nucleic acid sequence that can be detected and/or measured by methods well known in the art and described herein that are suitable for detecting the labeled moiety that is present on a labeled fragment.

As used herein, "endonuclease" refers to an enzyme that cleaves bonds, preferably phosphodiester bonds, within a nucleic acid molecule. An endonuclease according to the invention can be specific for singletstranded or double-stranded DNA or RNA.

As used herein, "exonuclease" refers to an enzyme that cleaves bonds, preferably phosphodiester bonds, between nucleotides one at a time from the end of a polynucleotide. An exonuclease according to the invention can be specific for the 5' or 3' end of a DNA or RNA molecule, and is referred to herein as a 5' exonuclease or a 3' exonuclease.

As used herein a "flap" refers to a region of single stranded DNA that extends from a double stranded nucleic acid molecule. A flap according to the invention is preferably between about 1–500 nucleotides, more preferably between about 5–25 nucleotides and most preferably between about 10–20 nucleotides.

In a preferred embodiment, the nucleic acid polymerase substantially lacks 5' to 3' exonuclease activity.

In a preferred embodiment, the cleavage structure comprises at least one oligonucleotide primer.

The invention also provides a method of detecting or measuring a target nucleic acid sequence comprising forming a cleavage structure by incubating a sample comprising a target nucleic acid sequence with a nucleic acid polymerase, cleaving the cleavage structure with a FEN nuclease to release a nucleic acid fragment, and detecting and/or measuring the release of the fragment as an indication of the presence of the target sequence in the sample.

As used herein, "detecting a target nucleic acid sequence" or "measuring a target nucleic acid sequence" refers to determining the presence of a particular target nucleic acid sequence in a sample or determining the amount of a particular target nucleic acid sequence in a sample as an indication of the presence of a target nucleic acid sequence in a sample. The amount of a target nucleic acid sequence that can be measured or detected is preferably about 1 molecule to 10^{20} molecules, more preferably about 100 molecules to 10^{17} molecules and most preferably about 1000 molecules to 10^{14} molecules. According to the invention, the detected nucleic acid is derived from the labeled 5' end of a

downstream probe of a cleavage structure according to the invention (for example C in FIG. 3), that is displaced from the target nucleic acid sequence by the 3' extension of an upstream probe of a cleavage structure according to the invention (for example A of FIG. 3). According to the present invention, a label is attached to the 5' end of the downstream probe (for example C in FIG. 3) comprising a cleavage structure according to the invention. Alternatively, a label is attached to the 3' end of the downstream probe and a quencher is attached to the 5' flap of the downstream probe. According to the invention, a label may be attached to the 3' end of the downstream probe (for example C in FIG. 3) comprising a cleavage structure according to the invention.

According to the invention, the downstream probe (for example C in FIG. 3) may be labeled internally. In a preferred embodiment, a cleavage structure according to the invention can be prepared by incubating a target nucleic acid sequence with a probe comprising a non-complementary, labeled, 5' region that does not anneal to the target nucleic acid sequence and forms a 5' flap, and a complementary 3' region that anneals to the target nucleic acid sequence. According to this embodiment of the invention, the detected nucleic acid is derived from the labeled 5' flap region of the probe. Preferably there is a direct correlation between the amount of the target nucleic acid sequence and the signal generated by the cleaved, detected nucleic acid.

As used herein, "detecting release of labeled fragments" or "measuring release of labeled fragments" refers to determining the presence of a labeled fragment in a sample or determining the amount of a labeled fragment in a sample. Methods well known in the art and described herein can be used to detect or measure release of labeled fragments. A method of detecting or measuring release of labeled fragments will be appropriate for measuring or detecting the labeled moiety that is present on the labeled fragments. The amount of a released labeled fragment that can be measured or detected is preferably about 25%, more preferably about 50% and most preferably about 95% of the total starting amount of labeled probe

As used herein, "labeled fragments" refer to cleaved mononucleotides or small oligonucleotides or oligonucleotides derived from the labeled cleavage structure according to the invention wherein the cleaved oligonucleotides are preferably between about 2–1000 nucleotides, more preferably between about 5–50 nucleotides and most preferably between about 16–18 nucleotides, which are cleaved from a cleavage structure by a FEN nuclease and can be detected by methods well known in the art and described herein

In a preferred embodiment, the nucleic acid polymerase substantially lacks 5' to 3' exonuclease activity.

In another preferred embodiment, the nucleic acid polymerase is a DNA polymerase.

In another preferred embodiment, the nucleic acid polymerase is thermostable.

As used herein, "thermostable" refers to an enzyme which is stable and active at temperatures as great as preferably between about 90–100° C. and more preferably between about 70–98° C. to heat as compared, for example, to a non-thermostable form of an enzyme with a similar activity. For example, a thermostable nucleic acid polymerase or FEN nuclease derived from thermophilic organisms such as *P. furiosus, M jannaschii, A. fulgidus* or *P. horikoshii* are more stable and active at elevated temperatures as compared to a nucleic acid polymerase from *E. coli* or a mammalian FEN enzyme. A representative thermostable nucleic acid polymerase isolated from *Thermus aquaticus* (Taq) is described in U.S. Pat. No. 4,889,818 and a method for using it in conventional PCR is described in Saiki et al., 1988, *Science* 239:487. Another representative thermostable nucleic acid polymerase isolated from *P. furiosus* (Pfu) is described in Lundberg et al., 1991, *Gene*, 108:1–6 Additional representative temperature stable polymerases include, e.g., polymerases extracted from the thermophilic bacteria *Thermus flavus, Thermus ruber, Thermus thermophilus, Bacillus stearothermophilus* (which has a somewhat lower temperature optimum than the others listed), *Thermus lacteus, Thermus rubens, Thermotoga maritima*, or from thermophilic archaea *Thermococcus litoralis*, and *Methanothermus fervidus*

Temperature stable polymerases and FEN nucleases are preferred in a thermocycling process wherein double stranded nucleic acids are denatured by exposure to a high temperature (about 95° C.) during the PCR cycle.

In another preferred embodiment, the FEN nuclease is a flap-specific nuclease.

In another preferred embodiment, the FEN nuclease is thermostable.

In another preferred embodiment, the cleavage structure is formed comprising at least one labeled moiety capable of providing a signal.

In another preferred embodiment, the cleavage structure is formed comprising a pair of interactive signal generating labeled moieties effectively positioned to quench the generation of a detectable signal, wherein the labeled moieties are separated by a site susceptible to FEN nuclease cleavage, thereby allowing the nuclease activity of the FEN nuclease to separate the first interactive signal generating labeled moiety from the second interactive signal generating labeled moiety by cleaving at the site susceptible to FEN nuclease, thereby generating a detectable signal.

In yet another preferred embodiment, the cleavage structure is formed comprising a hairpin-forming oligonucleotide probe having secondary structure.

As used herein, "secondary structure" refers to the conformation (for example a hairpin, a stem-loop structure, an internal loop, a bulge loop, a branched structure, or a pseudoknot) of a nucleic acid molecule wherein a sequence comprising a first single stranded sequence of bases followed by a second complementary sequence in the same molecule folds back on itself to generate an antiparallel duplex structure wherein the single stranded sequence and the complementary sequence anneal by the formation of hydrogen bonds. A "secondary structure" also refers to the conformation of a nucleic acid molecule comprising an affinity pair, wherein the affinity pair reversibly associates as a result of attractive forces that exist between the moieties. As used herein, "secondary structure" refers to a nucleic acid conformation which prevents probe binding to a capture element

As used herein, a "hairpin structure" or a "stem" refers to a double-helical region formed by base pairing between adjacent, inverted, complementary sequences in a single strand of RNA or DNA.

As used herein, "stem loop" structure refers to a hairpin structure, further comprising a loop of unpaired bases at one end.

In another preferred embodiment, the pair of interactive signal generating moieties comprises a quencher moiety and a fluorescent moiety.

In another preferred embodiment, the cleavage structure comprises at least one oligonucleotide primer.

The invention also provides a polymerase chain reaction process for detecting a target nucleic acid sequence in a sample comprising providing a cleavage structure, providing a set of oligonucleotide primers wherein a first primer contains a sequence complementary to a region in one strand of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand, and a second primer contains a sequence complementary to a region in a second strand of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand, and amplifying the target nucleic acid sequence employing a nucleic acid polymerase as a template-dependent polymerizing agent under conditions which are permissive for PCR cycling steps of (i) annealing of primers required for amplification to a template nucleic acid sequence contained within the target sequence and annealing primers required for formation of a cleavage structure to a target nucleic acid sequence, (ii) extending the primers wherein the nucleic acid polymerase synthesizes a primer extension product, and (iii) cleaving the cleavage structure employing a FEN nuclease as a cleavage agent for release of labeled fragments from the cleavage structure thereby creating detectable labeled fragments; and (d) detecting and/or measuring the release of labeled fragments as an indication of the presence of the target nucleic acid sequence in the sample.

The invention provides for a polymerase chain reaction process wherein amplification and detection of a target nucleic acid sequence occur concurrently (i.e. real time detection). The invention also provides for a polymerase chain reaction process wherein amplification of a target nucleic acid sequence occurs prior to detection of the target nucleic acid sequence (i.e. end point detection).

As used herein, an "oligonucleotide primer" refers to a single stranded DNA or RNA molecule that can hybridize to a nucleic acid template and primes enzymatic synthesis of a second nucleic acid strand. Oligonucleotide primers useful according to the invention are between about 10 to 100 nucleotides in length, preferably about 17–50 nucleotides in length and more preferably about 17–45 nucleotides in length. Oligonucleotide probes useful for the formation of a cleavage structure according to the invention are between about 17–40 nucleotides in length, preferably about 17–30 nucleotides in length and more preferably about 17–25 nucleotides in length. Oligonucleotide probes, as used in the present invention include oligonucleotides comprising secondary structure, including, but not limited to molecular beacons, safety pins (FIG. 10), scorpions (FIG. 11), and sunrise/amplifluor probes (FIG. 12), the details and structures of which are described below and in the corresponding figures.

As used herein, "template dependent polymerizing agent" refers to an enzyme capable of extending an oligonucleotide primer in the presence of adequate amounts of the four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP and dTTP) or analogs as described herein, in a reaction medium comprising appropriate salts, metal cations, appropriate stabilizers and a pH buffering system. Template dependent polymerizing agents are enzymes known to catalyze primer- and template-dependent DNA synthesis, and possess 5' to 3' nuclease activity. Preferably, a template dependent polymerizing agent according to the invention lacks 5' to 3' nuclease activity.

As used herein, "amplifying" refers to producing additional copies of a nucleic acid sequence, including the method of the polymerase chain reaction.

In a preferred embodiment, the nucleic acid polymerase substantially lacks 5' to 3' exonuclease activity.

In a preferred embodiment, the oligonucleotide primers of step b of the polymerase chain reaction process described above are oriented such that the forward primer is located upstream of a cleavage structure according to the invention and the reverse primer is located downstream of a cleavage structure according to the invention. The reverse primer is complementary to the opposite strand of the forward primer which is complementary to a strand of the cleavage structure.

In another preferred embodiment, the nucleic acid polymerase is a DNA polymerase.

In another preferred embodiment, the nucleic acid polymerase is thermostable.

In another preferred embodiment, the nucleic acid polymerase is selected from the group consisting of Taq polymerase and Pfu polymerase.

In another preferred embodiment the FEN nuclease is thermostable.

In another preferred embodiment the FEN nuclease is a flap-specific nuclease.

In another preferred embodiment the FEN nuclease is selected from the group consisting of FEN nuclease enzyme derived from *Archaeglobus fulgidus, Methanococcus jannaschii, Pyrococcus furiosus*, human, mouse or *Xenopus laevis*. A FEN nuclease according to the invention also includes *Saccharomyces cerevisiae* RAD27, and *Schizosaccharomyces pombe* RAD2, Pol I DNA polymerase associated 5' to 3' exonuclease domain, (e.g *E coli, Thermus aquaticus* (Taq), *Thermus flavus* (Tfl), *Bacillus caldotenax* (Bca), *Streptococcus pneumoniae*) and phage functional homologs of FEN including but not limited to T4 RNaseH, T5 5' to 3' exonuclease, T7 gene 6 exonuclease and T3 gene 6 exonuclease.

Preferably, only the 5' to 3' exonuclease domains of Taq, Tfl and Bca FEN nuclease are used.

In another preferred embodiment, the labeled cleavage structure is formed by the addition of at least one labeled moiety capable of providing a signal.

The invention also provides a polymerase chain reaction process for simultaneously forming a cleavage structure, amplifying a target nucleic acid sequence in a sample and cleaving the cleavage structure comprising: (a) providing an upstream oligonucleotide primer complementary to a region in one strand of the target nucleic acid sequence and a downstream labeled probe complementary to a region in the same strand of the target nucleic acid sequence, wherein the upstream primer contains a sequence complementary to a region in one strand of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand, and the downstream probe contains a sequence complementary to a region in a second strand of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand; and (b) detecting a nucleic acid which is produced in a reaction comprising amplification of the target nucleic acid sequence and cleavage thereof wherein a nucleic acid polymerase is a template-dependent polymerizing agent under conditions which are permissive for PCR cycling steps of (i) annealing of primers to a target nucleic acid sequence, (ii) extending the primers of step (a) wherein the nucleic acid polymerase synthesizes primer extension products, and wherein the primer extension product of the primer of step (a) partially displaces the downstream probe of step (a) to form a cleavage structure; and (iii) cleaving the cleavage structure employing a FEN nuclease as a cleavage agent for release of labeled fragments from the cleavage structure thereby creating detectable labeled fragments.